EFFECTS OF DIOXIN ON CLOCK, MELATONIN BIOSYNTHETIC, AND REDOX SENSITIVE GENES ON CHICK PINEALOCYTES *IN VITRO*

A Senior Scholars Thesis

by

JAYME HUNT

Submitted to the Office of Undergraduate Research
Texas A&M University
In partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2006

Major: Biology

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ABSTRACT

Effects of Dioxin on Clock, Melatonin Biosynthetic, and Redox Sensitive Genes On Chick Pinealocytes *In Vitro* (April 2006)

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It has been speculated that dioxin treatment can modulate circadian rhythms. Cytochrome P4501A (CYP1A) is strongly induced by the xenobiotic dioxin via activation of arylhydrocarbon receptors within cells. Furthermore, regulation of CYP1A has been shown to occur via redox dependent activity of nuclear factor 1 X-type (NF1X) and cellular redox potential can regulate the circadian clock. As such we hypothesized that exposure to dioxin could affect circadian rhythmicity. We explored the pharmacological effects of the environmental toxicant dioxin on the expression of canonical clock genes, genes involved in melatonin biosynthesis, and putative redox sensitive genes CYP1A5 (an avian ortholog of human CYP1A) and NF1X using quantitative real-time PCR analysis. Dioxin altered the amplitude and phase of the rhythms of clock genes Brain and Muscle ARNT-Like (BMAL) 1 and BMAL2; melatonin biosynthetic pathway genes, hydroxyindole-O-methyltransferase (HIOMT),

arylalkylamine-N-acetyltransferase (AANAT), and tryptophan hydroxylase (TrH); and redox sensitive genes, CYP1A5 and NF1X. These results suggest dioxin treatment may uncouple the melatonin biosynthetic pathway from the pineal circadian clock or that the circadian affects of dioxins may be mediated via its affects directly on melatonin synthesis and not directly at the level of the molecular circadian clock.

DEDICATION

This thesis is dedicated to my parents Terry and Cathy Hunt, who have encouraged me to succeed by putting hard work and determination into everything I strive for.

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CHAPTER I

INTRODUCTION[1]

Dioxin is an environmental contaminant that causes a wide variety of biochemical responses. It lowers levels of the pineal hormone melatonin in rats (Korkalainen et al., 2004) and increases melatonin metabolism by hepatocytes in fish (Pesonen et al., 2000). The toxic effects of dioxin are mediated through the aryl hydrocarbon receptor (Ah receptor), which belongs to the class of Period-ARNT-SIM (PAS) domain proteins (Pesonen et al., 2000). PAS proteins are transcription factors that can recognize and adapt to environmental changes (Korkalainen et al., 2004). The three members included in the family of PAS domain proteins are Period, which is involved in circadian rhythm generation, ARNT (Ah Receptor nuclear translocator), which is involved in xenobiotic metabolism, and SIM (single-minded protein), which regulates developmental functions. The cytoplasmic Ah receptor binds dioxin, heterodimerizes with ARNT, and translocates into the nucleus. This complex then activates transcription of the cytochrome P4501A (CYP1A) gene and other Ah receptor-

[1] This thesis follows the style and format of *Journal of Biological Rhythms.*

responsive genes by binding to their specific recognition sequences (Korkalainen et al., 2004). CYP1A is reported to be strongly induced by the xenobiotic dioxin via activation of arylhydrocarbon receptors within cells (Zhang et al., 2006). Furthermore, regulation of CYP1A has been shown to occur via redox dependent activity of nuclear factor 1 X-type (NF1X) (Morel et al., 1998). Interestingly, cellular redox potential can also regulate the circadian clock (Rutter et al., 2002).

Circadian rhythms are driven by interacting positive and negative feedback loops which make up the circadian molecular clock. Positive elements of the feedback loop include CLOCK, BMAL1, and BMAL2 genes. Their products activate transcription by binding to E box enhancers in the promoters of other genes, such as those in the promoters of arylalkylamine-N-acetyltransferase (AANAT), the rate-limiting enzyme in the melatonin biosynthetic pathway, and Period and Cryptochrome, the negative elements in the canonical clockwork loop. The circadian molecular clock is self-regulating in such a way that the positive elements activate the transcription of the negative elements and the negative elements inhibit positive element transcription. This shuts down the actions of the positive elements, including the transcription of the negative elements. Once the negative elements are degraded subsequent to

phosphorylation, positive elements are no longer inhibited and the process can repeat itself (Reppert and Weaver, 2002).

The chick pinealocyte culture is an *in vitro* model system. It is an isolated circadian pacemaker that can be easily manipulated and its biochemical output, melatonin, can be easily measured. Recently, a study utilizing a chick pineal-specific microarray has identified NF1X as a candidate gene that may play a role in clock function (Karaganis et al., submitted).

We hypothesized that dioxin will affect clock gene expression, most likely through interactions with the period gene since it is a PAS protein, with BMAL1 since it is potentially a dimerization partner for Ah Receptor, and that melatonin levels will be reduced, as has been observed in the rat (Korkalainen et al., 2004). We also hypothesized that transcription of CYP1A will be increased after dioxin exposure, due to its pivotal role in xenobiotic metabolism.

CHAPTER II

MATERIALS AND METHODS

Cell Culture

Newly hatched chicks were acquired (Hyline Hatching, Bryan, TX) and their pineal glands removed following decapitation. The pineal glands were digested with trypsin, and the dispersed cells plated evenly in 12-well polystyrene tissue culture plates. The cultures were grown for two days in McCoy's 5A modified medium supplemented with 10% chicken serum, 10% fetal bovine serum, and 1% PSN antibiotic cocktail (Invitrogen) in a humidified incubator at 37ºC with 5% CO_2 under a 12-hour light:dark cycle (Karaganis et al., submitted). The following two days, cells were given either glycerol in McCoy's 5A modified medium as a vehicle control or $5x10^{-6}$ M dioxin dissolved in the vehicle in McCoy's 5A modified medium. After the second day of treatment, media was collected every six hours starting two hours after lights came on. The collected media was frozen and stored for melatonin analysis. Cells were harvested in Trizol (Invitrogen) every six hours, at Zeitgeber Time (ZT) 2, 8, 14, and 20. The

Trizol samples were collected, homogenized, and then frozen at –80ºC for future RNA extraction. Two biological replicates were taken for each timepoint.

Quantitative Real-Time PCR Analysis

RNA was extracted from the homogenized tissues using an RNeasy kit (Qiagen), treated with DNase to eliminate any trace genomic DNA, primed with random hexamers, and cDNA was synthesized by reverse transcription using a Superscript II RT PCR kit (Invitrogen). The relative quantity of selected genes was measured by performing SYBR green-based quantitative real-time PCR using an ABI Prism 7700 Sequence Detection instrument (Applied Biosystems).

Standard curves were constructed for target gene cDNA and for cyclophilin, which was used as an endogenous control reference. All genes were analyzed in triplicate for each timepoint. Cyclophilin was chosen because its expression is not rhythmic across the day, and it is not acutely induced following dioxin exposure. The expression levels of the target genes were normalized to the endogenous reference values, and then normalized to a calibrator sample, composed of a mixture of cDNA from each timepoint of the vehicle samples. Each plate included a “no template control”

reaction, where cDNA was replaced with water, and an “RT- control” reaction, where reverse transcriptase enzyme was replaced with water, to rule out any genomic contamination (Karaganis et al., submitted).

CHAPTER III

RESULTS

Melatonin Biosynthetic Genes

The amplitudes and phases were altered in all three melatonin biosynthetic genes, hydroxyindole-O-methyltransferase (HIOMT); AANAT; and tryptophan hydroxylase (TrH), after dioxin treatment. The expression level of HIOMT was highest at ZT 8, but dioxin reduced the amplitude of expression without altering the phase. Normally, AANAT expression peaks at ZT 20, but dioxin increased the phase of the rhythm so that it peaked at ZT 14, without changing the amplitude. The expression level of TrH is highest at ZT 14, and dioxin increased the amplitude without altering the phase (Figure 1).

Clock Genes

The expression levels of some clock genes were affected by dioxin treatment whereas the expressions of others were not. Unaffected genes included cry1, per2, per3, and CLOCK, with only BMAL1 and BMAL2 affected by dioxin treatment. In controls,

the expression level of BMAL1 is highest at ZT14; following treatment with dioxin, the amplitude is increased but the phase is unaltered. BMAL2 peak expression is normally at ZT 20, but dioxin advanced the phase of this peak by 6 hours while also increasing the amplitude (Figures 2 and 3).

CYP1A5 and NF1X

Dioxin greatly affected the amplitude of CYP1A5 and NF1X. The amplitude of CYP1A5 was greatly reduced after dioxin treatment, and it now appears arrhythmic. In controls, the expression level of CYP1A5 is greatest at ZT 2, and following dioxin treatment, peak expression occurred at ZT 14. This shows that after dioxin treatment, there was a 2½-fold increase in the expression level of CYP1A5 during the early evening and night times. Normal expression levels of NF1X peak at ZT 8, but after dioxin treatment, the amplitude is greatly reduced and it no longer appears rhythmic (Figure 4).

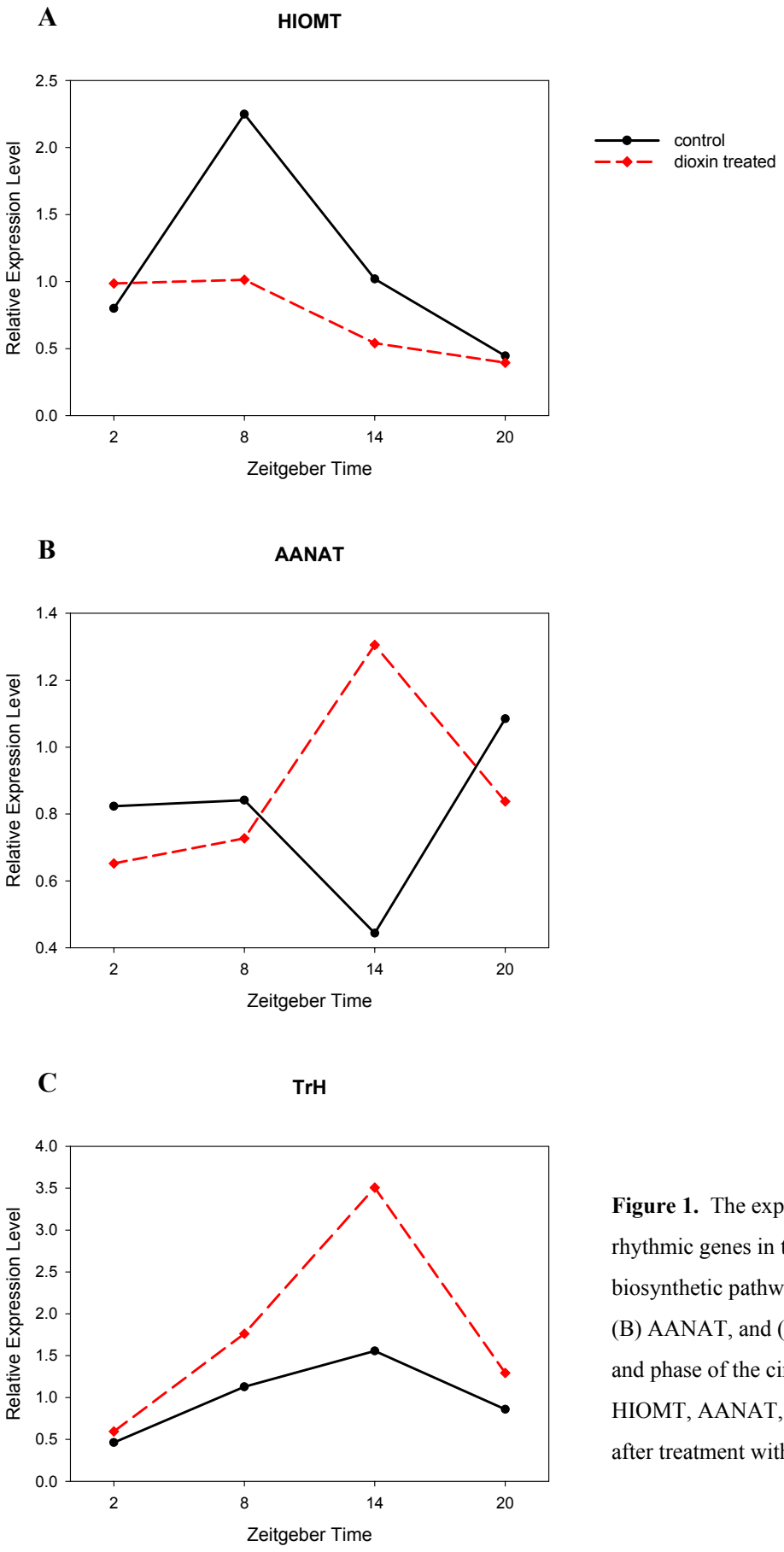

Figure 1. The expression levels of the rhythmic genes in the melatonin biosynthetic pathway *in vitro*. (A) HIOMT, (B) AANAT, and (C) TrH. The amplitude and phase of the circadian expression of HIOMT, AANAT, and TRH are altered after treatment with dioxin.

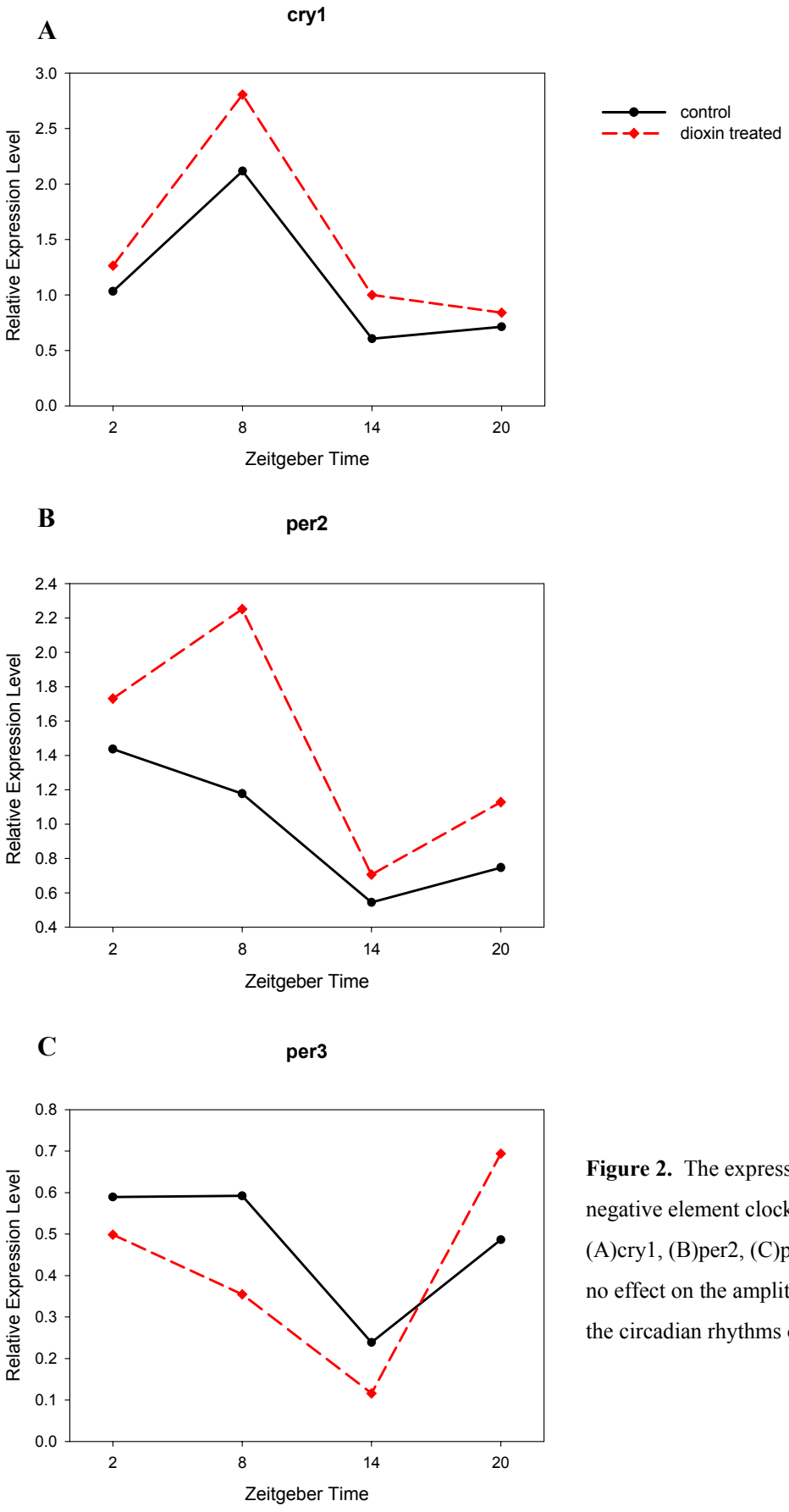

Figure 2. The expression levels of negative element clock genes *in vitro*. (A)cry1, (B)per2, (C)per3. Dioxin has no effect on the amplitude or phase of the circadian rhythms of cry1, per2, per3.

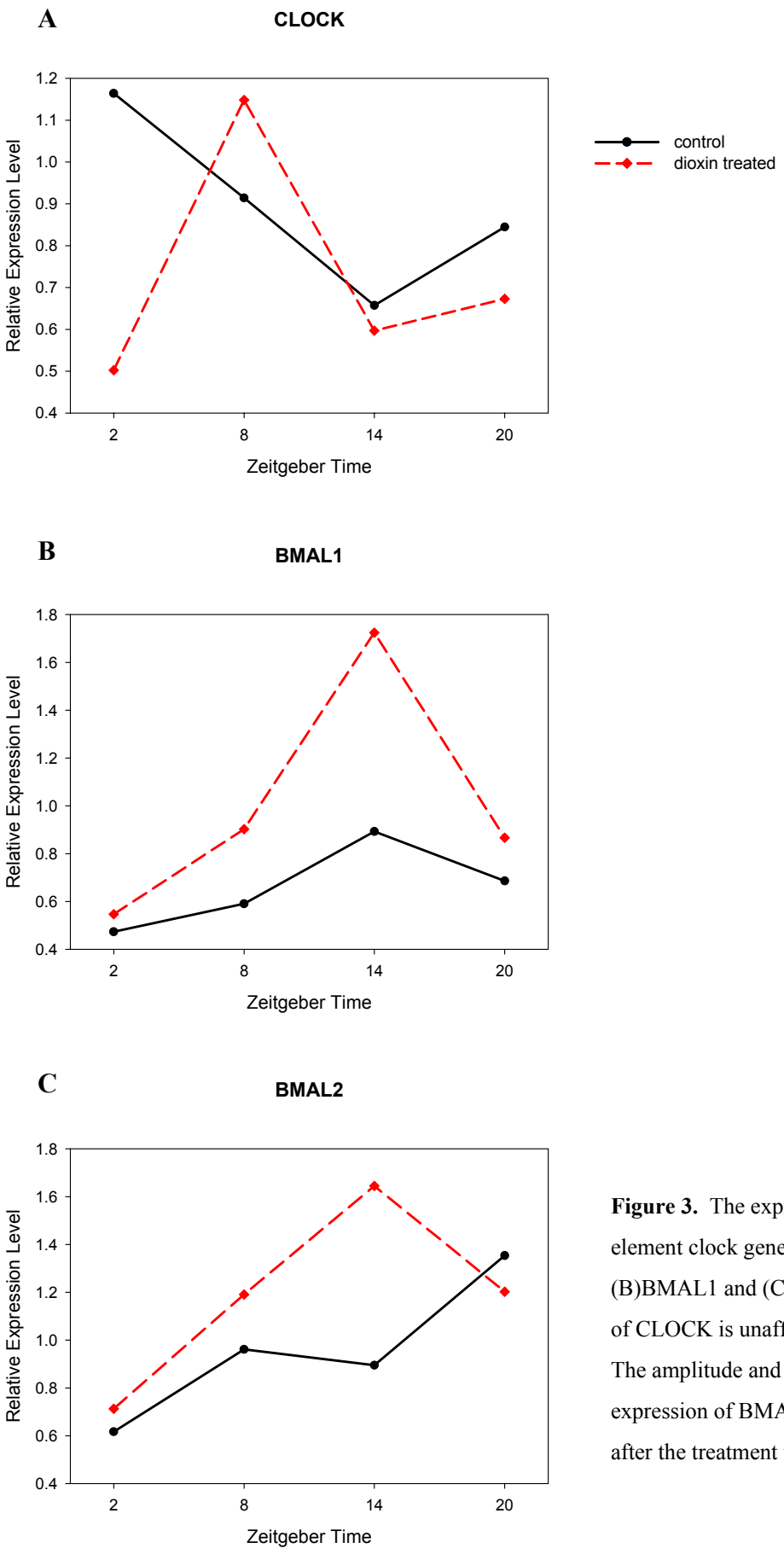

Figure 3. The expression levels of positive element clock genes *in vitro*. (A)CLOCK, (B)BMAL1 and (C)BMAL2. The expression of CLOCK is unaffected by dioxin treatment. The amplitude and phase of the circadian expression of BMAL1 and BMAL2 are altered after the treatment with dioxin.

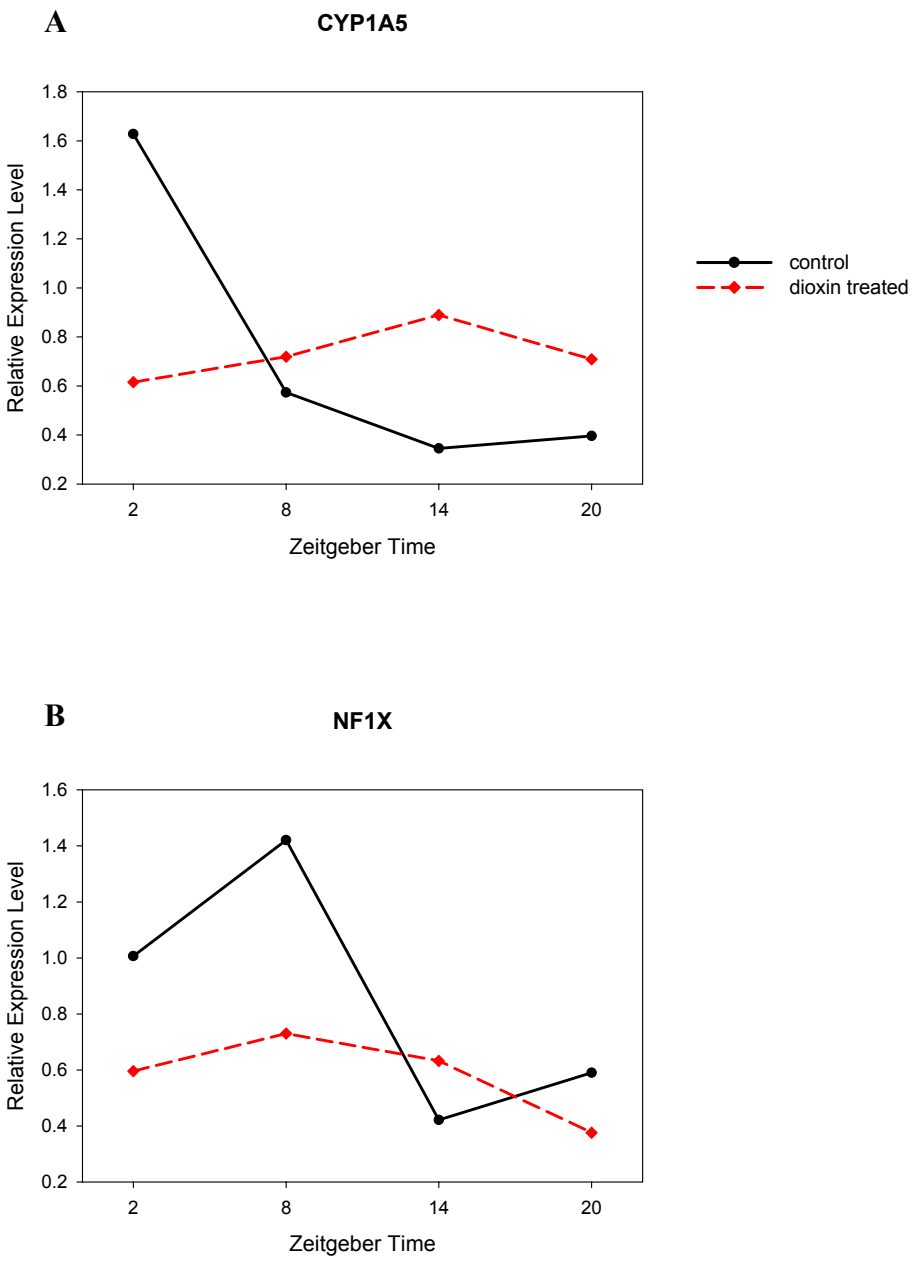

Figure 4. The expression levels of redox sensitive genes *in vitro*. (A)CYP1A5 and (B)NF1X. The phase and amplitude of the circadian expression of CYP1A5 and NF1X are altered after the treatment with dioxin.

CHAPTER IV

DISCUSSION

Our data suggest that the major effects of dioxin on the circadian system may be mediated through its effects on the melatonin pathways and not necessarily at the level of the molecular circadian clock, since the expression of most clock genes was unaffected by dioxin, and the expression of melatonin biosynthesis genes was greatly reduced and altered in phase by dioxin. Our data did not show that dioxin reduced peak levels of melatonin, as has been shown in the rat *in vivo* (Korkalainen et al., 2004), suggesting that increased melatonin metabolism after dioxin treatment may regulate melatonin levels, as has been demonstrated in trout hepatocytes *in vitro* (Pesonen et al., 2000). Future studies addressing dioxin's effects on the circadian clock should be conducted *in vivo* and incorporate the measurement of serum melatonin levels to see if overall melatonin levels are reduced.

Our study observed the expression of clock, melatonin biosynthetic, and redox sensitive genes at the mRNA level, however, mRNA must be translated and undergo further post-translational modifications in order to be functional. Future studies need to

be conducted to measure the protein levels of the respective gene products after dioxin treatment to see how dioxin affects their expression, since proteins ultimately comprise the enzymes that catabolize the reactions to produce outputs of melatonin. Post-translational modifications, such as degradation or phosphorylation, could also alter ultimate enzymatic activity.

Most canonical clock genes showed no change in expression after dioxin treatment, however the phase and amplitude of BMAL1 and BMAL2 were affected following dioxin treatment. It is possible that BMAL1 and BMAL2 expression levels can be quasi-independent from the rest of the molecular circadian clock. The observed increase in amplitude of BMAL expression might possibly be regulated by separable interactions with the Ah Receptor and are independent of those interactions with the circadian clock. Other *in vitro* studies have shown that redox reactions can regulate the circadian clock (Rutter et.al, 2002). The reduced forms of the cofactors, NADH and NADPH, were observed to activate CLOCK:BMAL1 heterodimer binding to DNA, and the oxidized forms of the cofactors, NAD+ and NADP+, inhibited DNA binding activity and promoted the homodimerization of BMAL1:BMAL1 (Rutter et al., 2002). This could be a possible explanation for why the expression level of BMAL1 increased

whereas other clock genes were unchanged in expression. Increased expression of BMAL1 could compensate for the greater trend towards BMAL1:BMAL1 homodimers by providing enough substrate for the proper number of CLOCK:BMAL1 heterodimers to form.

We also observed changes in the expression of the redox sensitive genes CYP1A5 and NF1X. The expression of CYP1A5 has been observed to increase after dioxin treatment in other species (Zhang et al., 2006). Also, in those studies, they focused primarily on expression of genes after dioxin treatment in liver cells whereas we focused on the pineal gland. In our study, expression of CYP1A5 was induced by dioxin, but only during the early evening and night. Other studies showed an increase in levels of CYP1A after dioxin treatment (Park et al., 1996), but in those studies, the time of day levels of CYP1A were measured was not taken into consideration. Furthermore, the length of time between exposure to dioxin and sampling can affect CYP1A levels, too. This could be a possible explanation for the reason our results do not line up with that of others. However, our results suggest that dioxin treatment abolishes the rhythmic expression of CYP1A5 which could lead to an impression of altered amplitude, depending upon the time of day expression levels were observed.

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